Soil Biology & Biochemistry 43 (2011) 2384–2396

Contents lists available at SciVerse ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Genetic diversity of rhizobia associated with indigenous legumes in different regions of Flanders (Belgium)

Sofie E. De Meyer[a], Koenraad Van Hoorde[a,b], Bram Vekeman[a], Tamara Braeckman[a], Anne Willems[a,*]

[a] Laboratory of Microbiology, Dept. of Biochemistry and Microbiology, Ghent University, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium
[b] Faculty of Applied Engineering Sciences, University College Ghent, Campus Schoonmeersen, Schoonmeersstraat 52, B-9000 Ghent, Belgium

ARTICLE INFO

Article history:
Received 10 June 2011
Received in revised form
8 August 2011
Accepted 13 August 2011
Available online 31 August 2011

Keywords:
Rhizobia
Indigenous legumes
Symbiotic genes *nodC* and *nifH*
Flanders
Belgium
Bradyrhizobium
Ensifer
Mesorhizobium
Rhizobium
Root nodules

ABSTRACT

We investigated the diversity of rhizobia isolated from different indigenous legumes in Flanders (Belgium). A total of 3810 bacterial strains were analysed originating from 43 plant species. Based on rep-PCR clustering, 16S rRNA gene and *recA* gene sequence analysis, these isolates belonged to *Bradyrhizobium*, *Ensifer (Sinorhizobium)*, *Mesorhizobium* and *Rhizobium*. Of the genera encountered, *Rhizobium* was the most abundant (62%) and especially the species *Rhizobium leguminosarum*, followed by *Ensifer* (19%), *Bradyrhizobium* (14%) and finally *Mesorhizobium* (5%). For two rep-clusters only low similarity values with other genera were found for both the 16S rRNA and *recA* genes, suggesting that these may represent a new genus with close relationship to *Rhodopseudomonas* and *Bradyrhizobium*. Primers for the symbiotic genes *nodC* and *nifH* were optimized and a phylogenetic sequence analysis revealed the presence of different symbiovars including genistearum, glycinearum, loti, meliloti, officinalis, trifolii and viciae. Moreover, three new *nodC* types were assigned to strains originating from *Ononis*, *Robinia* and *Wisteria*, respectively. Discriminant and MANOVA analysis confirmed the correlation of symbiosis genes with certain bacterial genera and less with the host plant. Multiple symbiovars can be present within the same host plant, suggesting the promiscuity of these plants. Moreover, the ecoregion did not contribute to the separation of the bacterial endosymbionts. Our results reveal a large diversity of rhizobia associated with indigenous legumes in Flanders. Most of the legumes harboured more than one rhizobial endosymbiont in their root nodules indicating the importance of including sufficient isolates per plant in diversity studies.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

Since the 19th century, the symbiosis between leguminous plants and bacteria collectively called rhizobia, is well known (As reviewed in: Dresler-Nurmi et al., 2007; Willems, 2006). Traditionally, most rhizobia species have been allocated to six genera in the Alphaproteobacteria: the fast to moderately fast growing genera *Rhizobium*, *Allorhizobium*, *Ensifer* (*Sinorhizobium*) and *Mesorhizobium*, the slow-growing genus *Bradyrhizobium* and the stem nodulating genus *Azorhizobium*. However, recent studies have reported the presence of other non-classical rhizobia belonging to the Betaproteobacteria (Chen et al., 2005; Vandamme et al., 2002), Gammaproteobacteria (Ibáñez et al., 2009; Muresu et al., 2010) and Actinobacteria (Palaniappan et al., 2010; Trujillo et al., 2010).

Nodulation and nitrogen fixation capacity of rhizobia are very important factors in understanding the symbiosis with legumes. The mechanisms behind this molecular interaction between the plant and the bacteria have been intensively studied (as reviewed in: Cooper, 2007; Downie, 2010; Masson-Boivin et al., 2009). Different genes are involved in the nodulation process, where the early nodulation genes *nodABC* are responsible for the core structure of the Nod factors. Therefore these genes are structurally and functionally conserved and found in most rhizobia studied to date (Dresler-Nurmi et al., 2007). The term biovar has been used in bacterial taxonomy to group strains of the same species with distinctive physiological characters. In rhizobia research it refers to strains symbiotic with the same host species. Recently Rogel et al. (2011) proposed to use the term symbiovar for strains distinguishable by their symbiotic capabilities and host plant. *Nif* and *fix* genes are responsible for the nitrogen fixation. N*ifH* is one of the most studied nitrogen fixing genes to date (Deng et al., 2011; Diouf et al., 2010; Mierzwa et al., 2010a; Rogel et al., 2011; Wdowiak-Wrobel and Malek, 2010). It encodes the two identical subunits of

* Corresponding author. Tel.: +32 9 2645103; fax: +32 9 2645092.
E-mail address: Anne.Willems@UGent.be (A. Willems).

0038-0717/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.soilbio.2011.08.005

dinitrogenase reductase and has proven to be useful for phylogenetic analysis (Chen et al., 2003; Dobert et al., 1994).

The family of leguminous plants is one of the major plant families in the world, comprising more than 17,000 species (APGIII, 2009). This family is divided into three subfamilies, the *Mimosoideae*, *Caesalpinioideae* and *Faboideae* (APGIII, 2009). The first two subfamilies consist of woody species with a more tropical distribution. The *Faboideae* is the largest subfamily and comprises woody and herbaceous species with a cosmopolitan distribution. The legume species in Belgium are restricted to this third subfamily and contain 102 plant species in 30 different genera (Lambinon et al., 1998). The flora of Flanders, the northern part of Belgium, is dominated by Atlantic and Mid-European species although some sub-boreal and sub-mediterranean species can be found (Van Landuyt et al., 2006a). Since Flanders is situated in the Atlantic biogeographical region, limited climate variation is present (Van Landuyt et al., 2011). The small-scale distribution of plant species is thus mainly dictated by geological and landscape elements, such as soil type and land-use system. Several characteristics were used to designate ecoregions as areas with a more or less uniform landscape, they include soil type, landscape morphology, land-use system, climate, topography and hydrology (Van Landuyt et al., 2006a). In Flanders, six ecoregions can thus be observed: dunes, polders, sandy-sandloamy region, campine, loamy region and the region of the Valley of the River Meuse (Van Landuyt et al., 2006a).

Legumes, including important crops such as beans, soybean, peanuts and clover are ecologically important plant species that can often grow on nutrient deficient soils, as a result of their associations with rhizobia that fix nitrogen. When the plants or the nodules decay, the fixed nitrogen is released in the soil and becomes available for other plants. This process improves soil structure and enables other plants to settle in this environment. Many studies have proven the usefulness of legumes in revegetation of arable lands (Freitas et al., 2010; Howieson et al., 2000; Rincon et al., 2008). However, because of this ecologic and economic importance, most studies have focused on cultivated legumes. Only a small portion of the rhizobial diversity present in wild legumes has been investigated, especially in indigenous legumes in Western Europe.

The aim of this study was to perform a systematic exploration of endosymbionts present in indigenous legumes in Flanders. Isolates obtained in several sampling campaigns were grouped and identified by using rep-PCR analysis, 16S rDNA and *recA* gene sequencing. Additionally, the phylogenies of the symbiosis genes *nodC* and *nifH* were investigated to assess the symbiotic capacity and the symbiovar type of the isolates. The focus, however, was on the typical rhizobia present in these indigenous legumes in Flanders, other isolates representing non-typical rhizobia will be reported in a separate study.

2. Materials and methods

2.1. Sampling

To organise the sampling campaigns we used the INBO grid (Research Institute for Nature and Forest) that divides Flanders into plots of 1 km^2 and is linked to a database that documents the plant species reported in each plot (Van Landuyt et al., 2006a). Plots were selected for sampling on the basis of the diversity of legume species and difference in ecoregion (campine, dune, polder, loamy and sandy-sandloamy region; Fig. 1). The sampling campaigns were performed over the summers of 2008 and 2009. In each selected plot one or two representative plants were sampled for each recognizable legume species. In most cases, whole plants were excavated and taken to the laboratory where the nodules were removed and preserved at 4 °C in tubes containing dried silica beads.

2.2. Isolation of rhizobia

The bacteria were isolated from surface-sterilized root nodules as follows: nodules were rehydrated for 30 min in sterile distilled

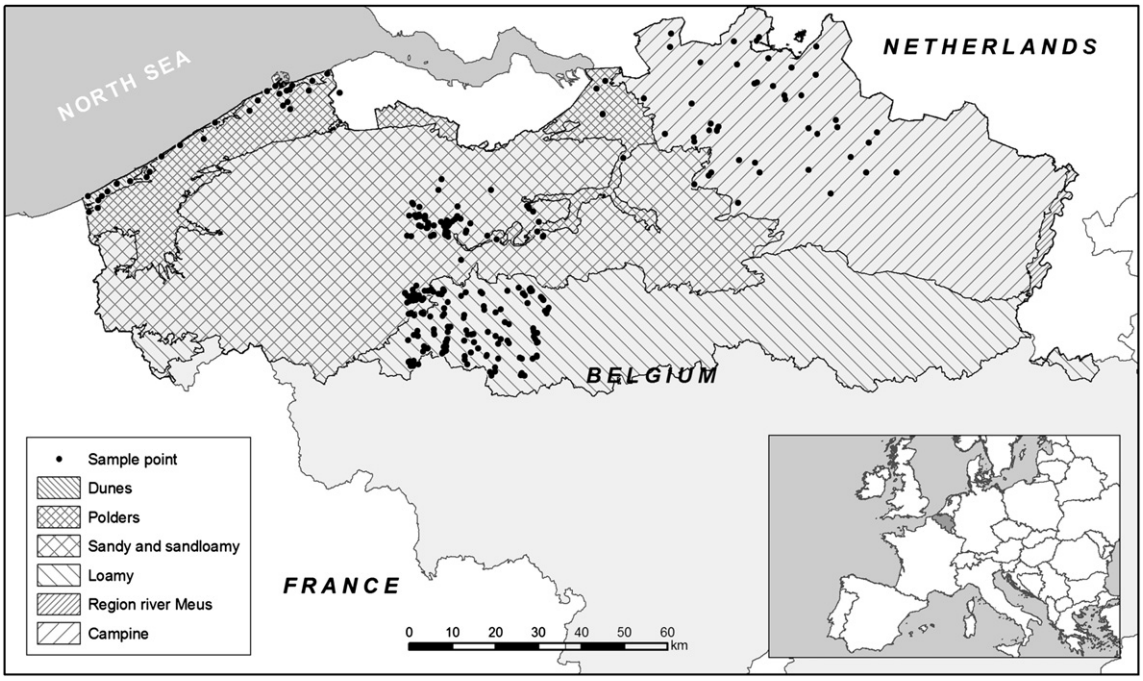

Fig. 1. Map of Belgium showing the sampling sites, marked as black dots, and the different ecoregions in Flanders, the northern part of Belgium.

water and vortexed in sterile distilled water with 0.5 mm sterile glass beads. They were surface sterilized by 3 min immersion in 3% sodium hypochlorite, afterwards the nodules were washed by immersion and vortexing in sterile distilled water with 0.5 mm sterile glass beads. To check the surface sterilization process, the surface-sterilized nodules were rolled over YMA agar plates (Vincent, 1970). The plates without bacterial growth were considered as successfully surface sterilized and isolates from these nodules were further used in the study. For isolation, the nodules were squashed in sterile physiological water (0.86% NaCl) and a dilution series was plated on YMA medium (Vincent, 1970). All YMA plates were incubated at 28 °C and regularly checked for growth up to 20 days. Two colonies of each morphological type were selected for isolation. The bacteria were purified by repeatedly streaking on YMA plates. All bacteria were stored in 15% glycerol + YMA broth tubes at −20 °C.

2.3. DNA extraction and genomic fingerprinting by $(GTG)_5$ rep-PCR

Genomic DNA of all isolates was prepared using the alkaline lysis method as described by Baele et al. (2000). Genomic fingerprint patterns were obtained for all strains using the repetitive extragenic palindromic (rep) PCR method with the GTG_5 primer (Gevers et al., 2001; Versalovic et al., 1994). The PCR was performed in a total volume of 25 μl for each reaction. The amplification products were separated by electrophoresis in 1.5% (w/v) agarose gel for 960 min at 55 V (constant voltage) in an incubator at 4 °C. The electrophoresed gels were stained for 30 min in a solution of 1 μg ml^{-1} EtBr in Tris acetic acid EDTA buffer (1×) and digital pictures were taken under UV light. The rep $(GTG)_5$ patterns were normalized and cluster analysis was performed using the software package BioNumerics v5.1 (Applied Maths, Sint-Martens-Latem, Belgium). Representatives of each rep-cluster analysed in this study are listed in Table 1 and Table S1.

2.4. Sequence analysis of 16S rRNA and recA gene

Nucleotide sequences of partial 16S rRNA (±350 bp) and *recA* (±500 bp) genes were obtained and used to determine the phylogenetic position of the strains. The primers and protocols used to amplify 16S rRNA and *recA* genes were described previously by Vancanneyt et al. (2004) and Martens et al. (2007), respectively. The acquired gene sequences were subjected to a FASTA search in the EMBL nucleotide sequence database to find the related species or genera (Pearson, 1990). All sequences from this study and the appropriate reference strains were aligned using the MEGA 5 software package (Tamura et al., in press). Phylogenetic trees were constructed using the Maximum Likelihood method, with the General time reversible model. Sequences of the 16S rDNA and *recA* genes determined in this study have been deposited in the EMBL database under the accession numbers: FR752819–FR753165 and FR774162–FR774192 for 16S rDNA; FR772356–FR772688 for the *recA* gene.

2.5. Sequence analysis of nodC and nifH gene

Nucleotide sequences of *nodC* (±550 bp) and *nifH* (±300 bp) genes were obtained using the primers and protocols listed in Table 2. The *nodC* primers were obtained from Sarite et al. (2005), with minor modifications to the reverse primer to broaden the range. To design the nifH439R primer we used *nifH* sequences derived from all publicly available complete genome sequences of strains belonging to the following genera: *Acidithiobacillus*, *Azoarcus*, *Azorhizobium*, *Azotobacter*, *Bradyrhizobium*, *Burkholderia*, *Chlorobaculum*, *Chlorobium*, *Chloroherpeton*, *Clostridium*, *Cupriavidus*, *Dehalococcoides*, *Desulfatibacillum*, *Desulfovibrio*, *Ensifer*, *Erwinia*, *Geobacter*, *Gluconacetobacter*, *Haliobacterium*, *Klebsiella*, *Leptothrix*, *Magnetospirillum*, *Mesorhizobium*, *Methylacidiphilum*, *Methylobacter*, *Methylocella*, *Methylococcus*, *Nostoc*, *Pelobacter*, *Pelodictyon*, *Polaromonas*, *Pseudomonas*, *Ralstonia*, *Rhodobacter*, *Rhodopseudomonas*, *Rhodospirillum*, *Rhizobium*, *Synechococcus*, *Syntrophobacter*, *Teredinibacter*, *Thermodesulfovibrio* and *Xanthobacter*. PCR amplification was performed using a Veriti 96-well Thermal cycler (Applied biosystems, Halle, Belgium). The reactions were performed in 50 μl mixtures and contained 5 μl DNA extract, 1U AmpliTaq DNA polymerase (Applied Biosystems), 200 μM of each GeneAmp dNTP (Applied Biosystems) and 0.5 μM of each primer in 1× PCR buffer (Applied Biosystems). The amplified products were purified using Nucleofast 96 PCR plates according to the manufacturer's instructions (Machery-Nagel, Düren, Germany). The purified DNA was sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI Prism 3130xl capillary sequencer, according to the manufacturer's instructions (Applied Biosystems). The *nodC* and *nifH* sequences obtained have been deposited in the EMBL database under the accession numbers: FR850942–FR851204 for the *nodC* gene and FR850690–FR850941 for the *nifH* gene.

2.6. Discriminant and MANOVA analysis

All data analyses were performed using BioNumerics v5.1 (Applied Maths). To visualise a correlation between the host plant, symbiovar or *nodC* type and ecoregion on the endosymbiont identity, discriminant analysis (DA) and multivariate analysis of variance (MANOVA) were performed. For this purpose a dataset was set up, comprising for each bacterial strains: the host plant, symbiovar or *nodC* type and the ecoregion where the host plants were collected. Moreover, the supporting *L* value (Wilk's Lambda likelihood ratio test) and *p* value (probability of the statistical significance) are mentioned for each discriminant.

3. Results

3.1. Sampling and isolation of rhizobia

The legumes sampled were identified as 42 species belonging to 17 genera: *Anthyllis*, *Cytisus*, *Galega*, *Laburnum*, *Lathyrus*, *Lotus*, *Lupinus*, *Medicago*, *Melilotus*, *Ononis*, *Ornithopus*, *Robinia*, *Securigera (Coronilla)*, *Trifolium*, *Ulex*, *Vicia* and *Wisteria* (Table S1). Among the host plants, only *Galega officinalis*, *Laburnum anagyroides*, *Lupinus polyphyllus*, *Medicago arabica*, *Robinia pseudoacacia*, *Securigera varia*, *Vicia villosa* and *Wisteria sinensis* were introduced plants, while the remaining plant species were indigenous in Belgium (according to Lambinon et al., 1998). Protected and endangered species were not sampled because the destruction of the plants could not be ruled out (Van Landuyt et al., 2006b). Priority was given to indigenous plant genera although some exotic genera were also included, since this gave the opportunity to compare the endosymbionts with native species.

Plants were collected from different ecoregions in Flanders (Fig. 1). These ecoregions are based on climatologic conditions, geology, geomorphology, soil type, groundwater and surface water content (Van Landuyt et al., 2006a) and thus also reflect soil texture, water content and nutrient availability, and may contribute to the presence or absence of some legume species in certain areas. For instance, *Trifolium fragiferum* requires sandy soils and is fairly salt tolerant; therefore we encountered and sampled this plant intensively in the coastal area. Other plant species such as *Trifolium pratense, Trifolium repens* and *Vicia cracca* do not demand specific conditions and were sampled in the majority of the sites. During the sampling a maximal diversity

Table 1
Species isolated, listing the representative strains, rep-clusters and number of isolates.

Bacterial species	Representative strains	Rep-clusters	# Isolates
Bradyrhizobium canariense	R-45721	Alone	1
Bradyrhizobium japonicum	R-45544, R-45545, R-45565, R-45575, R-45576, R-45618, R-45695, R-45696, R-45699, R-45700, R-45703, R-45710, R-45771, R-45948, R-45949, R-45953, R-45955, R-46014, R-46022, R-46023, R-46136	Alone, 10, 38, 39, 42, 43, 49, 50, 51, 53, 54, 56, 58, 59, 62, 67, 266, 278	255
Bradyrhizobium sp.	R-45547, R-45555, R-45574, R-45579, R-45657, R-45661, R-45662, R-45722, R-45743, R-45821, R-45954, R-45957, R-46016, R-46017, R-46021, R-46172, R-46173, R-46206, R-46210, R-46211, R-46212, R-46220, R-46224, R-46227, R-46277, R-46304, R-46306, R-46310, R-46311, R-46313, R-46151	Alone, 14, 40, 41, 44, 46, 52, 57, 60, 61, 63, 71, 73, 74, 103, 127, 184, 212, 240, 267	263
Ensifer kummerowiae	R-45578, R-45587	15, 389	5
Ensifer medicae	R-45556, R-45626, R-45627, R-45712, R-45718, R-45719, R-45730, R-45736, R-45742, R-45753, R-45777, R-45845, R-45881, R-45883, R-45895, R-45900, R-45924, R-45962, R-45965, R-45966, R-45996, R-46054, R-46127, R-46128, R-46130, R-46135, R-46148, R-46180, R-46201	Alone, 84, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 201, 202, 203, 204, 205, 208, 214, 215, 366, 436	371
Ensifer meliloti	R-45542, R-45584, R-45590, R-45643, R-45714, R-45790, R-45831, R-45832, R-45876, R-46047, R-46048, R-46049, R-46050, R-46055, R-46150, R-46153, R-46187, R-46189, R-46225, R-46272, R-46281, R-46309, R-46316	Alone, 1, 124, 125, 285, 343, 382, 384, 385, 386, 387, 388, 390, 391, 392, 394, 395, 396, 397, 399	323
Ensifer sp.	R-45905, R-46116, R-46170, R-46177	183, 186, 200, 446	20
Mesorhizobium loti	R-46072, R-45994	Alone, 108	5
Mesorhizobium sp.	R-45568, R-45615, R-45553, R-45567, R-45754, R-45755, R-45768, R-45817, R-45834, R-45843, R-45863, R-45888, R-45975, R-45976, R-46042, R-46043, R-46044, R-46045, R-46071, R-46074, R-46108, R-46110, R-46209, R-46253, R-46321, R-46328	Alone, 8, 25, 78, 81, 85, 86, 87, 88, 89, 92, 93, 95, 96, 106, 107, 109, 128, 176, 319, 430, 452	174
Rhizobium alamii	R-45988	Alone	1
Rhizobium cellulosilyticum	R-45828	Alone	1
Rhizobium giardinii	R-46131	Alone	1
Rhizobium leguminosarum	R-45611, R-45739, R-45740, R-45836, R-45891, R-45915, R-45557, R-45558, R-45559, R-45561, R-45562, R-45569, R-45572, R-45597, R-45598, R-45600, R-45601, R-45602, R-45603, R-45605, R-45612, R-45613, R-45619, R-45625, R-45634, R-45636, R-45639, R-45646, R-45668, R-45707, R-45715, R-45720, R-45725, R-45726, R-45727, R-45729, R-45735, R-45741, R-45747, R-45749, R-45750, R-45751, R-45760, R-45764, R-45765, R-45769, R-45770, R-45773, R-45778, R-45779, R-45780, R-45781, R-45782, R-45783, R-45784, R-45788, R-45791, R-45797, R-45803, R-45825, R-45844, R-45846, R-45847, R-45848, R-45849, R-45854, R-45855, R-45868, R-45869, R-45870, R-45871, R-45872, R-45873, R-45874, R-45877, R-45878, R-45879, R-45882, R-45884, R-45886, R-45887, R-45894, R-45896, R-45897, R-45899, R-45908, R-45909, R-45911, R-45914, R-45916, R-45917, R-45919, R-45920, R-45921, R-45923, R-45929, R-45931, R-45932, R-45935, R-45937, R-45964, R-45967, R-45968, R-45969, R-45970, R-45971, R-45978, R-45979, R-45981, R-45983, R-45984, R-45985, R-45986, R-45995, R-45999, R-46000, R-46001, R-46002, R-46003, R-46005, R-46009, R-46059, R-46061, R-46077, R-46082, R-46083, R-46084, R-46086, R-46090, R-46091, R-46092, R-46094, R-46095, R-46096, R-46097, R-46098, R-46099, R-46101, R-46102, R-46104, R-46105, R-46106, R-46107, R-46109, R-46112, R-46114, R-46118, R-46119, R-46120, R-46121, R-46122, R-46123, R-46125, R-46133, R-46137, R-46139, R-46154, R-46160, R-46161, R-46168, R-46175, R-46178, R-46179, R-46186, R-46190, R-46191, R-46199, R-46204, R-46205, R-46207, R-46214, R-46215, R-46217, R-46232, R-46236, R-46237, R-46249, R-46250, R-46256, R-46262, R-46267, R-46270, R-46273, R-46274, R-46286, R-46287, R-46290, R-46293, R-46294, R-46295, R-46296, R-46297, R-46298, R-46299, R-46303, R-46315, R-46317, R-46324, R-46325, R-46327	Alone, 9, 13, 76, 113, 114, 115, 116, 119, 121, 122, 123, 131, 132, 133, 134, 135, 136, 137, 138, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 154, 155, 156, 157, 158, 159, 160, 162, 163, 164, 165, 166, 167, 168, 174, 175, 182, 216, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 230, 231, 232, 233, 235, 237, 239, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 259, 260, 261, 262, 263, 264, 265, 268, 269, 270, 271, 274, 276, 277, 286, 287, 288, 289, 290, 292, 294, 295, 297, 298, 299, 300, 301, 302, 306, 307, 309, 310, 311, 315, 316, 328, 330, 331, 332, 334, 340, 342, 344, 345, 346, 348, 349, 350, 351, 352, 353, 354, 355, 356, 358, 359, 360, 363, 364, 365, 367, 368, 369, 372, 373, 374, 405, 407, 409, 417, 441	2235
Rhizobium radiobacter	R-45711	Alone	1
Rhizobium sp.	R-45591, R-45595, R-45609, R-45614, R-45621, R-45648, R-45666, R-45724, R-45761, R-45802, R-45818, R-45819, R-45829, R-45835, R-45860, R-45889, R-45989, R-45990, R-45992, R-46039, R-46040, R-46140, R-46158, R-46165, R-46166, R-46218, R-46234, R-46235, R-46247, R-46248, R-46254, R-46258, R-46260, R-46261, R-46265, R-46266, R-46271, R-46283, R-46284	Alone, 27, 65, 66, 80, 94, 117, 153, 173, 279, 283, 291, 296, 304, 305, 308, 318, 327	131
Alphaproteobacteria sp.	R-45974, R-45977	21, 22	23

of plant species was harvested. In total, isolates were obtained from 655 plants from 43 plant species.

One nodule per sampled plant was selected and used for isolation. In total, 4464 isolates were obtained and used for further analysis. The present report covers 3810 of these that were found to be traditional rhizobia. The remaining 654 isolates will be reported in a separate study on non-rhizobial endophytes.

Table 2
Oligonucleotide primers and PCR cycling conditions used for *nodC* and *nifH* sequence analysis.

Primer	Position	Sequence 5′-3′	PCR cycling	Reference
nifHF1	28–54	TAY GGN AAR GGN GGN ATY GGN AAR TC	5 min 95 °C, 3 × (1 min 95 °C, 2 min 15 s 60 °C, 1 min 15 s 72 °C), 30 × (35 s 95 °C, 1 min 15sec 60 °C, 1 min 15 s 72 °C), 5 min 72 °C	Dedysh et al., (2004)
nifH439R	439–419	GGC ATN GCR AAN CCD CCR CA		This study
nodC540F	544–566	TG ATY GAY ATG GAR TAY TGG CT	5 min 95 °C, 3 × (1 min 95 °C, 2 min 15 s 50 °C, 1 min 15 s 72 °C), 30 × (35 s 95 °C, 1 min 15sec 50 °C, 1 min 15 s 72 °C), 5 min 72 °C	Sarita et al. (2005)
nodC1164R	1164–1184	GAY ARC CAR TCG CTR TTG		Modified from Sarita et al. (2005)

3.2. Rep $(GTG)_5$ PCR fingerprinting

Rep-PCR was performed to identify duplicate isolates, to reduce the number of strains and to get a first idea on the extent of diversity sampled. High resolution rep-profiles were generated of all test strains. A Pearson correlation/UPGMA analysis was performed and clusters were delineated at 80%. To analyse the reproducibility of this technique, one control strain was included each time and these repeats clustered together with correlation levels of 90.8%–98.3%, which is in line with previously reported data (Gevers et al., 2001). At 80% correlation, a total of 279 rep-clusters were obtained and 80 strains occupied isolated positions (Table S1), revealing a high level of diversity. With regard to the number of colonies selected for purification, two of each morphology type seems reasonable since these duplicates always grouped within the same rep-cluster. In total, 386 strains were used for further genetic identification.

3.3. Identification of rhizobia based on 16S rRNA gene and recA housekeeping gene

The identification process started with the sequencing of the first part of the 16S rRNA gene (±350 bp) to obtain a genus identification. Subsequently, the *recA* gene was sequenced to obtain species identification (Table S1). For a minority of strains (13.7%) the primers used failed to amplify the *recA* gene and their identification was therefore limited to genus level on the basis of the partial 16S rDNA. In general, the phylogeny of the *recA* housekeeping gene was similar to that of the 16S rDNA and a species identification could be derived (Table S1). *Rhizobium* was the most abundant genus found (62.2%), followed by *Ensifer* (18.9%), *Bradyrhizobium* (13.6%) and *Mesorhizobium* (4.7%). Noticeable is the presence of 58.7% *Rhizobium leguminosarum* among the strains tested, in line with the large number of *Trifolium* and *Vicia* plants that were sampled (see below). All other rhizobia were present in less prominent numbers.

The 519 *Bradyrhizobium* isolates were divided into 52 rep-clusters. These represent *Bradyrhizobium canariense*, *Bradyrhizobium japonicum* and unnamed *Bradyrhizobium* species. In *Ensifer*, 791 isolates were divided into 48 rep-clusters. These clusters are closely related to *Ensifer kummerowiae*, *Ensifer medicae*, *Ensifer meliloti* or unnamed *Ensifer* sp. *E. medicae* is the most abundant with 51.6% of all *Ensifer* isolates. The 179 *Mesorhizobium* strains were divided into 28 rep-clusters, a minority represent *Mesorhizobium loti* isolates and the rest correspond to unnamed new groups within *Mesorhizobium*. The 2370 *Rhizobium* strains were divided into 196 rep-clusters. These clusters correspond to *Rhizobium alamii*, *Rhizobium cellulosilyticum*, *Rhizobium giardinii*, *R. leguminosarum*, *Rhizobium radiobacter* and unnamed *Rhizobium* species. The *R. leguminosarum* clusters account for 94.3% of all *Rhizobium* strains and this species is therefore the most abundant representative of this genus. For two strains (belonging to rep-clusters 21 and 22) the similarity values were very low with known genera and they may represent a new genus related to *Rhodopseudomonas* and *Bradyrhizobium* (Table 1, Table S1, Fig. S1).

3.4. Hostplant – endosymbiont association – symbiovar/nodC type

For the different host plant species, an overview of the endosymbionts is shown in Fig. 2. To verify the potential nodulation

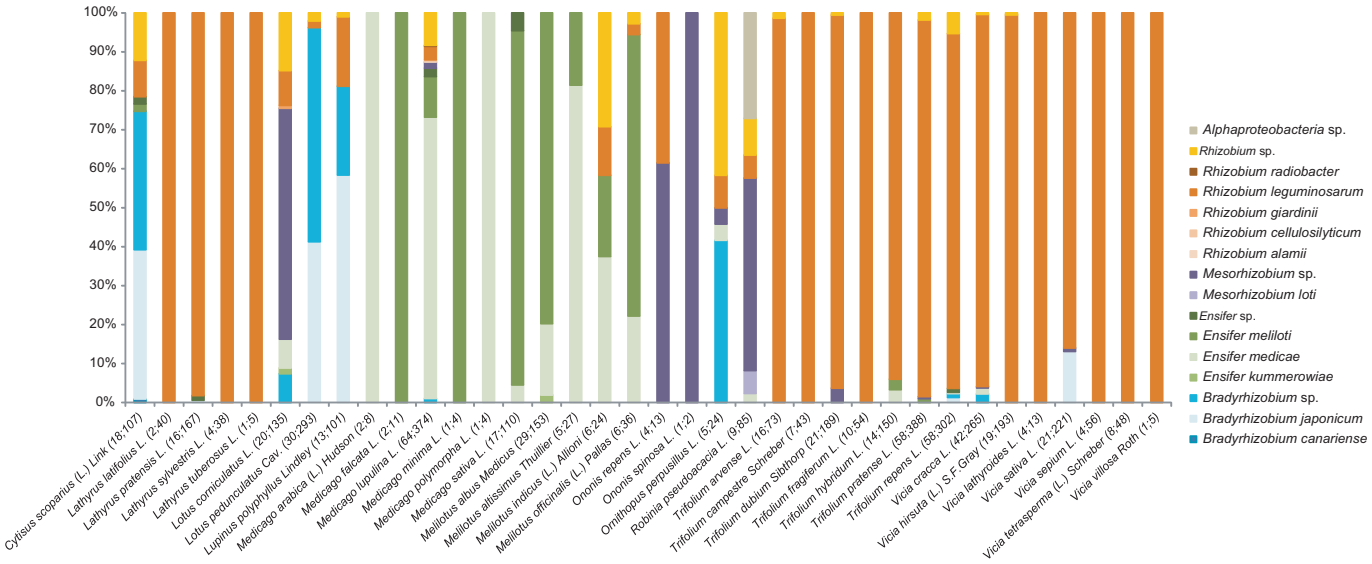

Fig. 2. Stacked column chart showing the host plant species with their rhizobial endosymbionts. Plant names are listed followed by in brackets the number of plants sampled and the number of isolates. corn. stands for corniculatus. The following plant species that were sampled less than three times and found to harbour only one endosymbiont species are therefore not represented in the figure: *Anthyllis vulneraria L.: Mesorhizobium sp.* (1;22), *Galega officinalis* L.: *Rhizobium* sp. (1;6), *Laburnum anagyroides Medicus*: *Bradyrhizobium* sp. (1;1), *Securigera varia* (L.) *Lassen*: *Rhizobium leguminosarum* (1;7), *Ulex europaeus* L.: *Bradyrhizobium japonicum*: (1;3) and *Wisteria sinensis* (Sims) *Sweet*: *Mesorhizobium* sp.(1;1).

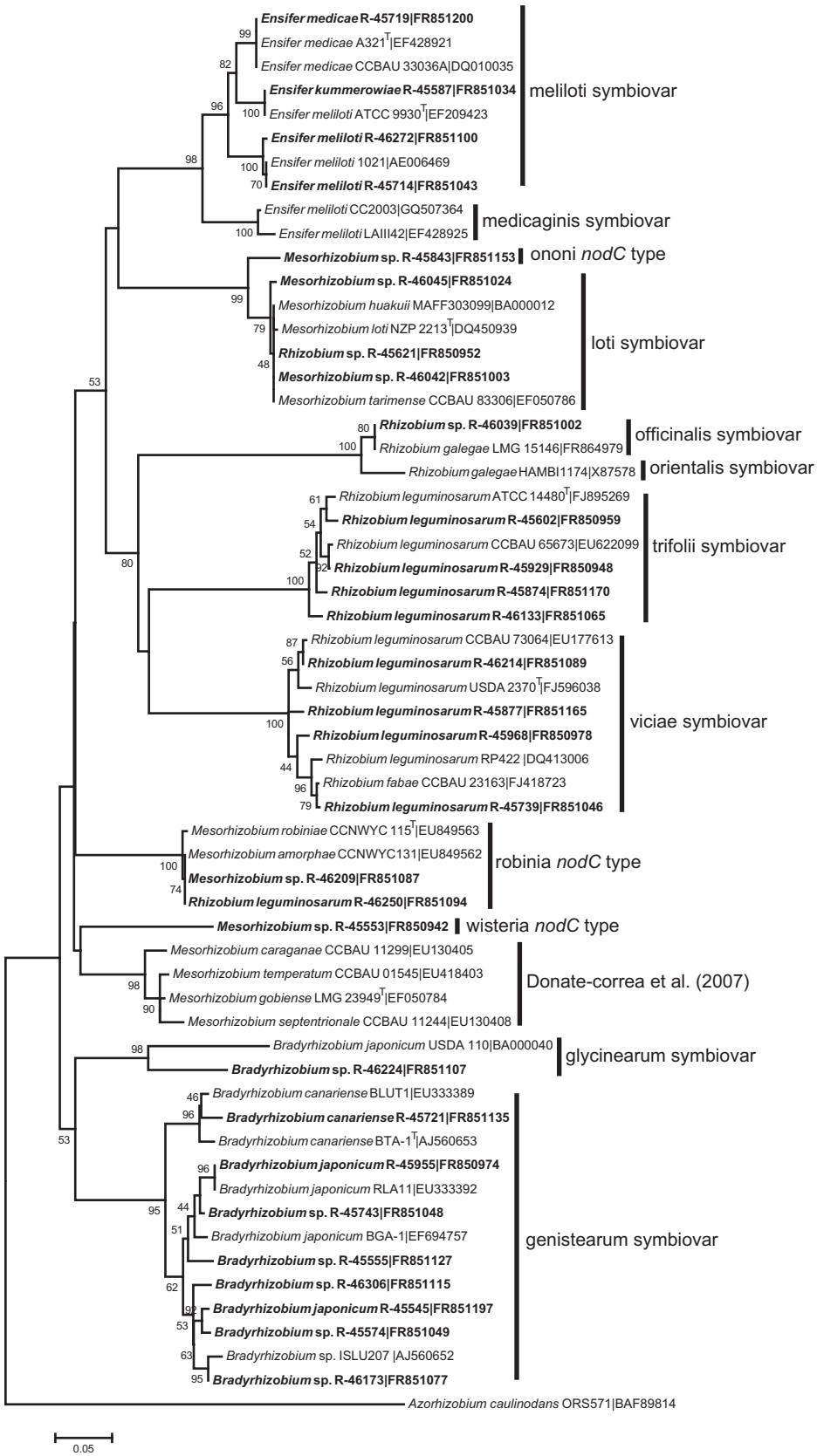

Fig. 3. Maximum likelihood tree based on the *nodC* sequences of representative rhizobial species. Strain number and sequence accession numbers are listed. Percentage bootstrap values based on 500 replicates are given at the nodes. The sequence of *Azorhizobium caulinodans* ORS571^{T} was included as an outgroup. Symbiovars or *nodC* types are indicated after the species name. Type strains are indicated with a superscript T and the strains isolated in this study are marked in bold. Bar, 5% substitution per nucleotide position.

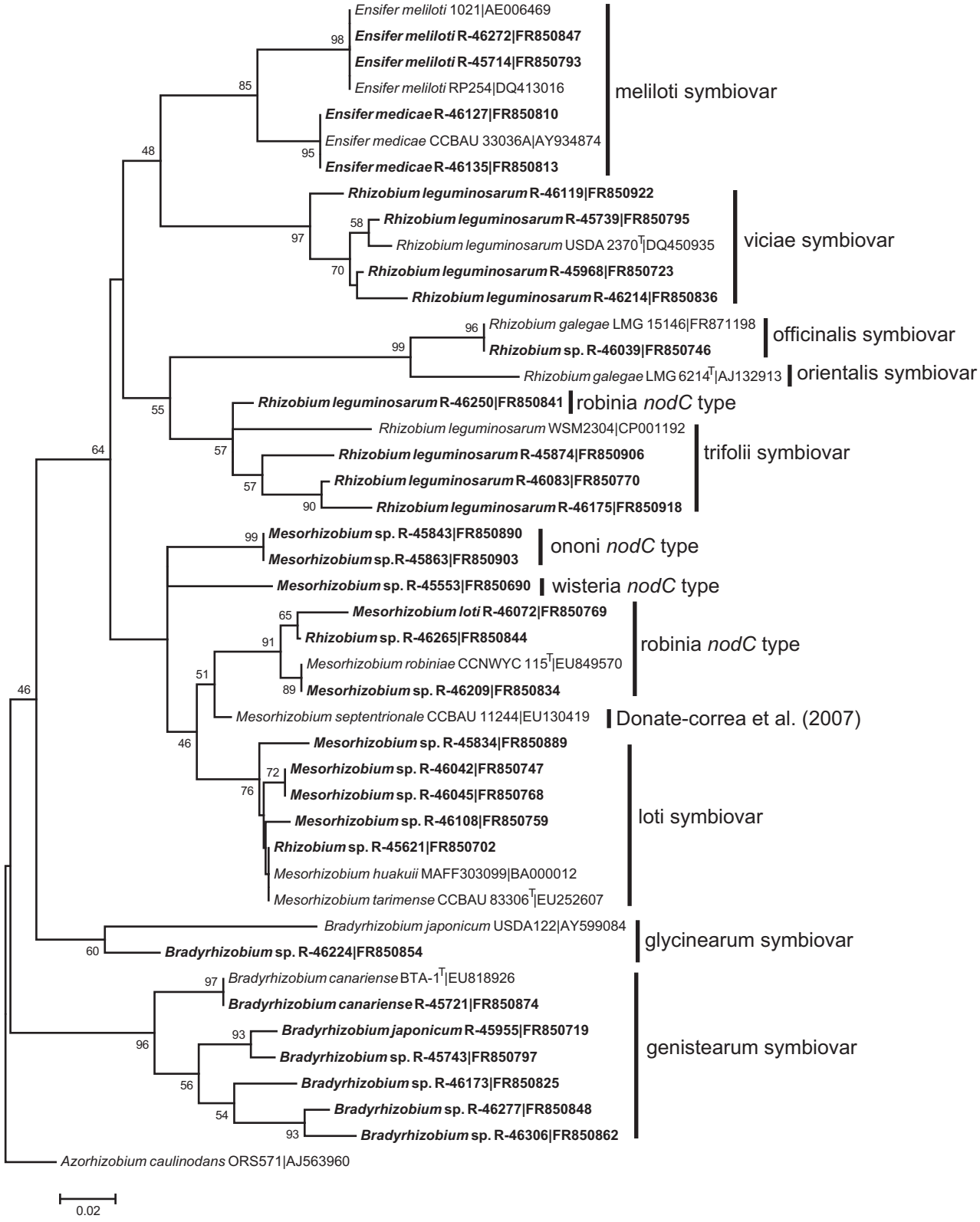

Fig. 4. Maximum likelihood tree based on the *nifH* sequences of representative rhizobial species. Strain number and sequence accession numbers are listed. Percentage bootstrap values based on 500 replicates are given at the nodes. The sequence of *Azorhizobium caulinodans* ORS571^{T} was included as an outgroup. Symbiovars or *nodC* types are indicated after the species name. Type strains are indicated with a superscript T and the strains isolated in this study are marked in bold. Bar, 5% substitution per nucleotide position.

and nitrogen fixation capacity of our isolates the *nodC* and *nifH* genes were analysed for representative strains (Table S1, Figs. 3 and 4). To optimize published *nodC* primers, small modifications were made to the reverse primer (Table 2). Moreover, one new *nifH* reverse primer was designed, since primers available in literature gave poor results (Table 2). For all strains the phylogenies of the *nodC* and *nifH* gene were the same, except for strain R-46250 (Figs. 3 and 4, Fig. S2). We used the system of symbiotic variants or symbiovars (equivalent to biovars) proposed by Rogel et al. (2011) to label certain clusters. When no symbiovar was available in literature, a *nodC* type was assigned to the corresponding strains. We did not assign new symbiovars because this would require nodulation tests that were not performed in this study. Six known symbiovars were identified in the *nodC* and *nifH* analysis (Figs. 3 and 4, Fig. S2, Table S1). The *Bradyrhizobium* species isolated in this study belong to two symbiovar types, glycinearum and genistearum proposed by Velazquez et al. (2010) and Vinuesa et al. (2005), respectively. The meliloti symbiovar cluster also comprises *E. medicae* strains with high bootstrap support in *nodC* and *nifH* phylogenies (Figs. 3 and 4). No symbiovar has been proposed by Rogel et al. (2011) for the *E. medicae* group and therefore we suggest that these strains could be part of the meliloti symbiovar according to the *nodC* and *nifH* sequence data (Figs. 3 and 4, Fig. S2). The loti symbiovar cluster grouped all strains isolated from *Lotus* host plants and other loti symbiovar strains from literature (Rogel et al., 2011). The strains nodulating *Trifolium* and *Vicia* species all clustered together with the trifolii and vicia symbiovars reported in literature (Rogel et al., 2011). Moreover, the viciae symbiovar group harboured strains isolated from host plants other than *Vicia*. Strains isolated from *Robinia pseudoacacia* and *Trifolium arvense* clustered together with strains isolated by Wei et al. (2009) from *Robinia pseudoacacia*. Therefore the name robinia is proposed for this *nodC* type. One strain (R-46250) from *Robinia pseudoacacia* has a *nodC* gene sequence identical to the strains represented by the robinia *nodC* type, but is different in its *nifH* gene sequence. Additionally, we proposed wisteria *nodC* type for *Mesorhizobium* sp. R-45553 isolated from *Wisteria sinensis*. *Mesorhizobium* sp. strains R-45843 and R-45863, both isolated from *Ononis* plant species represent a separate branch in the *nodC* and *nifH* gene tree and therefore the ononi *nodC* type is proposed (Figs. 3 and 4, Fig. S2). *Rhizobium* sp. strain R-46039 has been isolated from *G. officinalis* plants and shows close relationship to the officinalis symbiovar.

3.5. Discriminant and MANOVA analysis

Discriminant and MANOVA analysis of the composite dataset comprising the bacterial isolates confirmed the discrimination of four different groups (Figs. 5 and 6). Analysis of the bacterial isolates in function of the ecoregion revealed no significant discrimination (data not shown). On the other hand, discriminative characters for the four groups (Figs. 5 and 6) could mainly be attributed to the symbiovar/*nodC* type and to a lesser degree to the host plant. The separation on the *X*-axis is well supported and a low *L* value ($L = 0.014$) and *p* value ($p = 0.001\%$) confirmed the significance of the discriminants meliloti, trifolii and viciae symbiovar and host plants *Medicago lupulina*, *Medicago sativa, Trifolium* spp. *and Vicia* spp. responsible for the separation of group 2 (*Ensifer*) and 4 (*Rhizobium*). Along the *Y* axis two separate clusters were obtained, characterised by genistearum and glycinearum symbiovars and host plants *Lotus pedunculatus* and *Cytisus scoparius*, supported by a medium *L* value ($L = 0.085$) and a low *p* value ($p = 0.001\%$). The *Z*-axis ($L = 0.407$; $p = 0.001\%$) was characterised by the loti symbiovar and host plants *Lotus corniculatus* and *Robinia pseudoacacia*.

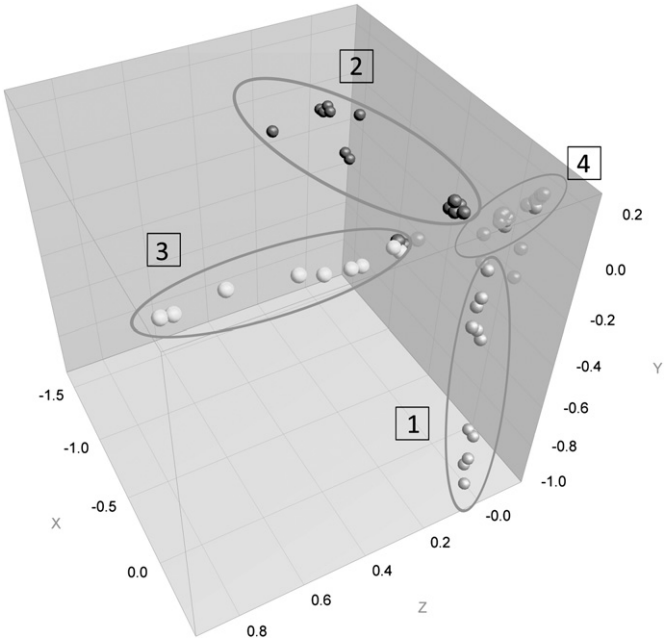

Fig. 5. Three-dimensional discriminant analysis plot showing the relative contributions of the three discriminants $X = 49\%$, $Y = 37\%$ and $Z = 14\%$. The bacterial isolates are plotted against the symbiovars/*nodC* types and the host plants.

4. Discussion

In this study the taxonomic and symbiotic diversity of typical rhizobia bacteria present in indigenous legumes in Flanders was explored. Phylogenetic analyses which are used to clarify the bacterial relationships are usually based on the comparison of the 16S rDNA sequences and were used in past rhizobia research (Eardly et al., 1992; Jarvis et al., 1997; Weisburg et al., 1991; Willems and Collins, 1993). Recently, housekeeping genes were additionally sequenced to obtain a more precise identification, since 16S rDNA is highly similar between many rhizobial species and on its own insufficient for species identification (Farida et al., 2009; Gaunt et al., 2001; Lorite et al., 2010; Martens et al., 2007; Menna et al., 2009; Rivas et al., 2009). In the current study, genus level identification was based on partial 16S rRNA gene sequences, whereas for species identification the *recA* gene sequences were analysed. This approach revealed the presence of bacteria belonging to the genera *Bradyrhizobium*, *Ensifer*, *Mesorhizobium* and *Rhizobium* (Table 1, Table S1). The grouping of a number of strains into clusters that are distinct from existing type species indicates they are possible new species. However, further polyphasic studies are required to characterize these strains and classify or describe them.

4.1. Host plant − endosymbiont species

Remarkable is the enormous amount of *R. leguminosarum* isolates found, comprising nearly 60% of all rhizobia (Table 1, Fig. 2). However, 49% of all sampled nodules were *Trifolium* and *Vicia* species and this to a large extent accounts for the high abundance of this species in our study. Nevertheless, rep-PCR revealed a large diversity among our *R. leguminosarum* strains (156 distinct rep-clusters) that could be due to a metabolic diversity of the strains as described by Wielbo et al. (2010). Additionally, no particular rep-cluster of these *R. leguminosarum* strains could be assigned to one specific host plant. *R. leguminosarum* is known for its broad host range (Weir et al., 2004) but has preferred associations with *Trifolium* and *Vicia* plants (Ramirez-Bahena et al., 2009). In this study

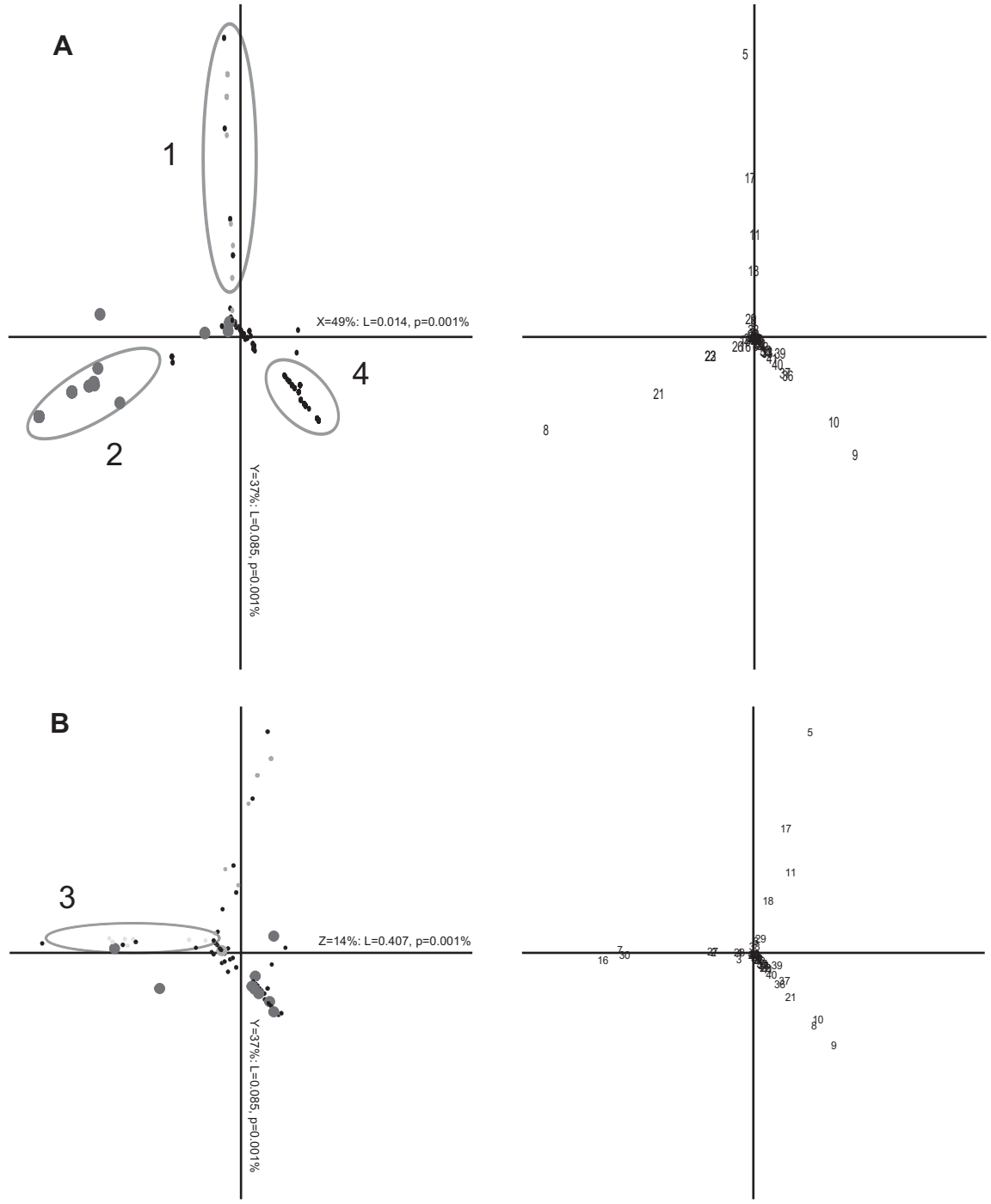

Fig. 6. Two-dimensional MANOVA plot showing the discriminants responsible for the separation of the isolates. A = separation according the *X* and *Y* axis, B = separation according to the *Z* and *Y* axis. The right panel represents the 'character' plot which maps the characters of the left panel, the bacterial isolates. Main discriminants = 5: genistearum symbiovar, 6: glycinearum symbiovar, 7: loti symbiovar, 8: meliloti symbiovar, 9: trifolii symbiovar, 10: viciae symbiovar, 11: *Cytisus scoparius*, 16: *Lotus corniculatus*, 17: *Lotus pedunculatus*, 18: *Lupinus polyphyllus*, 21: *Medicago lupulina*, 22: *Medicago sativa*, 30: *Robinia pseudoacacia*, 36: *Trifolium pratense*, 37: *Trifolium repens*, 39: *Vicia cracca* and 40: *Vicia hirsuta*. ●: *Ensifer*, ●: *Rhizobium*, ●: *Bradyrhizobium*, ●: *Mesorhizobium*.

R. leguminosarum was indeed found in nodules of *Trifolium* and *Vicia* plant species but also in *C. scoparius*, *Lathyrus latifolius*, *Lathyrus pratensis*, *L. corniculatus*, *L. polyphyllus*, *M. lupulina*, *Melilotus indicus*, *Robinia pseudoacacia*, and *Securigera varia*. Alvarez-Martinez et al. (2009) reported that endosymbionts of *Vicia* species from different countries belong to a phylogenetically compact group, indicating that these legumes are restrictive hosts. In the present study, *R. leguminosarum* accounts for 95% and 72% of the endosymbionts present in *Trifolium* and *Vicia,* respectively. Little diversity in the nodule endosymbionts is thus present, as suggested in previous reports (Alvarez-Martinez et al., 2009).

C. scoparius, *Ornithopus perpusillus* and *Robinia pseudoacacia* seem to be promiscuous hosts for root nodule endosymbionts (Fig. 2). *C. scoparius* has been studied intensively and is known for its associations with *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium* species (Lafay and Burdon, 2006; Sajnaga et al., 2001; Weir et al., 2004). In this

study 75% of the *C. scoparius* isolates belonged to *Bradyrhizobium* with *B. japonicum* as the major endosymbiont (Fig. 2). Surprisingly no *Mesorhizobium* isolates were found in this study, however we did find *E. meliloti* (2% of the *Cytisus* isolates), which to our knowledge has never before been reported in *C. scoparius*.

The endosymbionts reported previously for *Ornithopus* species are *M. loti* and multiple *Bradyrhizobium* species (Jarabo-Lorenzo et al., 2003; Jarvis et al., 1997; Vinuesa et al., 2005). In our study, *O. perpusillus* harboured 50% *Rhizobium* and especially *R. leguminosarum* in its nodules. Noteworthy, is the higher amount of *Rhizobium* compared to *Bradyrhizobium* (42%) isolates, the latter being the typical endosymbiont of *Ornithopus* (Bottomley et al., 1994; Jarabo-Lorenzo et al., 2003). Moreover, *E. medicae* (4.2%) was recovered for the first time from *O. perpusillus* root nodules.

Robinia pseudoacacia originated from North America and was first recorded in Europe in the 17th century. In Belgium this plant gained popularity as a useful ornamental garden plant. Ulrich and Zaspel (2000) provided the first insights in the phylogenetically diverse endosymbionts of *Robinia* and confirmed that indigenous rhizobia may be associated with this plant. Recent studies reported the presence of various *Mesorhizobium* species and indicated that they might be the typical rhizobia for this plant (Mierzwa et al., 2009, 2010b; Wei et al., 2009). In our study, we indeed recovered 55% *Mesorhizobium* isolates, but further analysis is necessary to identify these isolates properly at species level. In addition, *Rhizobium* (15%), *E. medicae* (3%) and a possible new genus (27%) closely related to *Rhodopseudomonas* and *Bradyrhizobium* were found (Fig. S1). Further study is ongoing using a polyphasic approach to classify these strains, including several phenotypic and biochemical tests, as well as phylogenetic analysis of housekeeping genes and DNA–DNA hybridisations.

Studies of root nodule bacteria from different *Lotus* species in an arid region of South Tunisia, in the Xinjiang desert soils of China, in Argentina and the Canary Islands reported rhizobia closely related to *Bradyrhizobium*, *Ensifer*, *Mesorhizobium* and *Rhizobium* (Estrella et al., 2009; Han et al., 2008; Lorite et al., 2010; Zakhia et al., 2004). We found *Mesorhizobium* to be the most abundant rhizobia genus in *L. corniculatus*. This is not unexpected since *Mesorhizobium* is the typical endosymbiont of *Lotus* species (Pankhurst et al., 1979). However, a large difference was noticed between the rhizobia of the two *Lotus* species investigated in this study (*L. corniculatus* and *L. pedunculatus*). The main endosymbionts were respectively, *Mesorhizobium* (59%) and *Bradyrhizobium* (96%), the latter being surprisingly abundant. Moreover, to our knowledge this is the first report on the presence of *E. kummerowiae*, *E. medicae* and *R. giardinii* in root nodules of endemic *Lotus* species in Western Europe. Nodulation tests are needed to assess the potential of these isolates to renodulate *Lotus* species as it cannot be excluded that these strains accidentally entered the host plant together with the regular endosymbiont.

As visualized in Fig. 2 *Medicago* and *Melilotus* are dominated by *Ensifer* endosymbionts, the natural endosymbionts of these plants (Bromfield et al., 2010; Fred et al., 1932; Merabet et al., 2010; Mnasri et al., 2009). Noticeable is the relatively limited diversity in endosymbionts in these two plant genera, whereas in previous studies different *Ensifer*, *Mesorhizobium* and *Rhizobium* species were recorded (Hou et al., 2009; Kan et al., 2007; Mnasri et al., 2009). Most species studied here only harbour *Ensifer* endosymbionts except *M. indicus* and *Melilotus officinalis* which harbour *R. leguminosarum* and *Rhizobium* sp. and *M. lupulina* where *Bradyrhizobium* sp., *Mesorhizobium* sp., *R. alamii*, *R. cellulocilyticum* and *R. radiobacter* were present in addition to *Ensifer*. *L. anagyroides* harboured *Bradyrhizobium* species and to our knowledge, this is the first study reporting the endosymbionts of this species. However, additional nodules of this plant should be investigated to confirm the presence of only *Bradyrhizobium* species as root nodule endosymbionts.

4.2. Symbiovar and nodC diversity

The nodulation and nitrogen fixation capacity are characters usually studied in rhizobia research, since they give an idea of symbiotic potential and host specificity (Diouf et al., 2010; Moulin et al., 2004; Perret et al., 2000). The effective contribution of particular bacteria towards nitrogen fixation should ideally be tested in plant inoculation studies. With many plant species and bacterial strains to be tested, we used *nodC* and *nifH* PCR amplification to study the presence of these genes as an indication of their symbiotic potential. Our results show that the phylogenies of the *nodC* and *nifH* genes were not entirely consistent with these of the 16S rRNA and *recA* genes, confirming different evolutionary histories of symbiotic and chromosomal genes as previously described (Dresler-Nurmi et al., 2007; Haukka et al., 1998; Steenkamp et al., 2008). Symbiotic genes may be transmitted by horizontal gene transfer, since they are often located on plasmids or transposable elements (Bailly et al., 2007; Raymond et al., 2004). For all strains the phylogenies of *nodC* and *nifH* were congruent, except for one strain, *R. leguminosarum* R-46250. This incongruence could be due to genetic rearrangements and/or horizontal gene transfer, as has been reported in the past in rhizobia symbiovars (Flores et al., 1988; Mergaert et al., 1997; Raymond et al., 2004; Suominen et al., 2001; Sy et al., 2001).

Symbiovars genistearum and glycinearum were described for bacteria nodulating genistoid plants (*Adenocarpus*, *Chamaecytisus*, *Cytisus, Lupinus, Spartocytisus* and *Teline*) and soybean plants, respectively (Velazquez et al., 2010; Vinuesa et al., 2005). In this study, *Bradyrhizobium* species isolated from *C. scoparius*, *L. anagyroides*, *Lotus pedunculatus, L. polyphyllus* and *O. perpusillus* clustered with either genistearum or glycinearum symbiovars (Figs. 3 and 4, Fig. S2, Supplementary table S1). Additionally, the separation of the two symbiovars glycinearum and genistearum was confirmed with DA and MANOVA analysis. Within the genistearum symbiovar, bacteria have been reported originating from *Ornithopus* species, but not from the plant genus *Lotus* (Vinuesa et al., 2005). Here we found symbiovar genistearum in *Lotus*, in line with the fact that *Lotus* and *Ornithopus* both belong to the Loteae tribe. The glycinearum symbiovar is typical for *Bradyrhizobium* species nodulating *Glycine max* from the Phaseolae tribe (Vinuesa et al., 2005). In our study we found *nodC* and *nifH* sequences of *Bradyrhizobium* isolates from *C. scoparius* (R-46224) and *L. anagyroides* (R-46310) grouping with *B. japonicum* symbiovar *glycinearum* USDA122 (Figs. 3 and 4). Both these plant genera belong to the genistoid tribe. Further studies investigating other symbiosis genes and nodulation tests are necessary to fully understand the relationship of our isolates with the glycinearum symbiovar isolates.

Different symbiovars, such as acaciae, acaciellae, ciceri, lancerottense, medicaginis, mediterranense, meliloti and sesbaniae have been proposed within the bacterial genus *Ensifer* (Ba et al., 2002; Boivin et al., 1997; Maâtallah et al., 2002; Mnasri et al., 2007; Rogel et al., 2011; Villegas et al., 2006). Each of them is characterised by different symbiosis genes and mostly restricted to a particular host plant. The *Ensifer* strains in this study originate from the plant genera *Cytisus*, *Lotus*, *Medicago, Melilotus, Ornithopus* and *Trifolium* and all belong to the meliloti symbiovar (Figs. 3 and 4, Fig. S2, Table S1). While *E. meliloti* is known to nodulate *Melilotus*, *Medicago* and *Trigonella*, we also found *E. meliloti* isolates from *C. scoparius* to belong to the meliloti symbiovar. Furthermore, *E. medicae* isolates from *O. perpusillus*, *L. corniculatus*, *T. repens* as well as *Melilotus albus* and several *Medicago* species also grouped in the symbiovar meliloti. This was also the case for *E. kummerowiae* isolates from *M. albus*. Moreover, this grouping was supported by DA and MANOVA revealing one group containing mostly *Ensifer* species and characterised by the meliloti symbiovar.

The *Mesorhizobium* strains isolated from *Anthyllis vulneraria* and *L. corniculatus* cluster together with *M. huakuii* MAFF 303099, which was assigned to the loti symbiovar by Turner et al. (2002). According to the *nodC* and *nifH* gene and amino acid sequence phylogenies (Figs. 3 and 4, Fig. S2) *Mesorhizobium* strains R-45843 and R-45863 from *Ononis repens* constituted a separate cluster for which the *nodC* type ononi is proposed.

As reported by Radeva et al. (2001) two symbiovars exist within the rhizobia isolated from *Galega orientalis* and *Galega officinalis*. Our strains isolated from *G. officinalis* plant species cluster together with the officinalis symbiovar, as expected.

R. leguminosarum strains isolated from several *Trifolium* plant species in this study, harbour similar *nodC* and *nifH* genes (Figs. 3 and 4, Fig. S2, Table S1) and could be assigned to the trifolii symbiovar, as they group together with trifolii symbiovar strains from literature. Within the viciae symbiovar group, much more variation is present in the host plant species. Certain strains isolated in this study from *Lathyrus*, *Medicago*, *Melilotus*, *Robinia*, *Vicia* and *Trifolium* plant species harbour similar symbiosis genes and cluster within the viciae symbiovar group. This has been reported before in literature and illustrates the complex origin of these symbiosis genes (Depret and Laguerre, 2008; Hynes and Oconnell, 1990). Moreover, it has been suggested that symbiovar viciae strains are not restricted to one particular host plant, but harbour promiscuous symbiosis genes (Mutch and Young, 2004). Discriminant and MANOVA analysis support these findings by revealing one group mainly comprising *Rhizobium* strains which is characterised by trifolii and viciae symbiovars.

On the comparison of indigenous and exotic host plants present in Flanders, only tentative conclusions could be drawn since some exotic plants were less intensively sampled. Rhizobia recovered from the introduced legume *G. officinalis* were found to be specific for this plant, since rhizobia present within these plants all grouped together in the same rep-clusters. Other exotic plants, such as *L. anagyroides*, *Lupinus polyphyllus, M. arabica*, *Robinia pseudoacacia, Securigera varia, Vicia villosa* and *Wisteria sinensis* were found not to be specific hosts. Rhizobia encountered within these exotic plants share rep-clusters with rhizobia encountered in indigenous legumes. Perez-Fernandez and Lamont (2003) found that indigenous Australian rhizobia were as effective in nodulating exotic and native legumes. However, Lafay and Burdon (2006) and Weir et al. (2004) found that exotic legumes harbour rhizobial lineages different from those nodulating native legumes. Our results indicate that certain plant species such as *G. officinalis* conform to the latter report and harbour specific rhizobia not found in other legumes. On the other hand all other exotic legumes investigated in this study tended to harbour rhizobia similar to those found in native legumes. This promiscuity could increase their ability to spread into new habitats. Some exotic host plants (*G. officinalis* and *Wisteria sinensis)* harbour a specific symbiovar or *nodC* type within their nodules, suggesting a specific interaction between the host plant and the endosymbionts. The other exotic legumes harboured one or more symbiovar/*nodC* types similar to indigenous legumes, enabling them to interact with multiple rhizobia.

Analysis of the bacterial isolates and symbiovars/*nodC* types in function of the ecoregion revealed no significant discrimination (data not shown). This may be explained by the fact that Flanders is a densely populated cross-roads for goods and people in Western Europe and that most of the sampled plants occurred almost everywhere in the Flanders region, irrespective of ecoregion.

4.3. Conclusion

Our study of endosymbionts present in nodules of native legumes in Flanders revealed a large diversity of rhizobia. Most of the legumes harboured more than one rhizobial endosymbiont in their root nodules indicating the importance of including sufficient isolates per plant in diversity studies. The overall majority of the isolates were identified as *Rhizobium* species with a dominance of *R. leguminosarum* although this was to a large extent due to the success of *Lathyrus*, *Trifolium* and *Vicia* species that were encountered in most of the sampled plots and particularly associate with this rhizobial species. We found *Mesorhizobium* to be the most abundant rhizobia genus in *L. corniculatus*. However, to our knowledge this is the first report on the presence of *E. kummerowiae*, *E. medicae* and *R. giardinii* in root nodules of endemic *Lotus* species in Western Europe. *C. scoparius*, *O. perpusillus* and *Robinia pseudoacacia* seem to harbour multiple symbiovars and/or *nodC* types, implying they are promiscuous hosts for root nodule endosymbionts. Different symbiovars were found among our isolates, including genistearum, glycinearum, loti, meliloti, officinalis, trifolii and viciae. Moreover, new *nodC* types ononi, robinia and wisteria were assigned to certain clusters. Discriminant and MANOVA analysis revealed the correlation of symbiosis genes with certain bacterial genera and less with certain host plants. For two rep-clusters only low similarity values with other genera were found for both the 16S rRNA and *recA* gene, suggesting that these may represent a new genus with close relationship to *Rhodopseudomonas* and *Bradyrhizobium*.

Acknowledgements

We thank the Special Research Fund (BOF)-UGent and the Foundation for Scientific Research (FWO)-Flanders for research grants. We thank the Research Institute for Nature and Forest (INBO) and the Natuurpunt organisation for facilitating access to nature reserves in Flanders. We are grateful to Prof. Paul Goetghebeur for assisting with the plant determination and the use of the Botanical Garden of the university, to Wouter Van Landuyt for supplying the INBO database and the ecoregion data. Special thanks to Prof. Philippe De Maeyer and Bart De Wit for supplying the sampling map (Fig. 1). The Research Fund University College Ghent is acknowledged for the postdoctoral fellowship of Dr. Koenraad Van Hoorde.

Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.soilbio.2011.08.005.

References

Alvarez-Martinez, E.R., Valverde, A., Ramirez-Bahena, M.H., Garcia-Fraile, P., Tejedor, C., Mateos, P.F., Santillana, N., Zuniga, D., Peix, A., Velazquez, E., 2009. The analysis of core and symbiotic genes of rhizobia nodulating *Vicia* from different continents reveals their common phylogenetic origin and suggests the distribution of *Rhizobium leguminosarum* strains together with *Vicia* seeds. Archives of Microbiology 191, 659–668.

APGIII, 2009. An update of the angiosperm phylogeny group classification for the orders and families of flowering plants: APG III. Botanical Journal of the Linnean Society 161, 105–121.

Ba, S., Willems, A., De Lajudie, P., Roche, P., Jeder, H., Quatrini, P., Neyra, M., Ferro, M., Prome, J.C., Gillis, M., Boivin-Masson, C., Lorquin, J., 2002. Symbiotic and taxonomic diversity of rhizobia isolated from *Acacia tortilis* subsp. *raddiana* in Africa. Systematic and Applied Microbiology 25, 130–145.

Baele, M., Baele, P., Vaneechoutte, M., Storms, V., Butaye, P., Devriese, L.A., Verschraegen, G., Gillis, M., Haesebrouck, F., 2000. Application of tRNA intergenic spacer PCR for identification of *Enterococcus* species. Journal of Clinical Microbiology 38, 4201–4207.

Bailly, X., Olivieri, I., Brunel, B., Cleyet-Marel, J.C., Bena, G., 2007. Horizontal gene transfer and homologous recombination drive the evolution of the nitrogen-fixing symbionts of *Medicago* species. Journal of Bacteriology 189, 5223–5236.

Boivin, C., Ndoye, I., Lortet, G., Ndiaye, A., De Lajudie, P., Dreyfus, B., 1997. The *Sesbania* root symbionts *Sinorhizobium saheli* and *S. teranga* bv. sesbaniae can form stem nodules on *Sesbania rostrata*, although they are less adapted to stem

nodulation than *Azorhizobium caulinodans*. Applied and Environmental Microbiology 63, 1040–1047.
Bottomley, P.J., Cheng, H.H., Strain, S.R., 1994. Genetic structure and symbiotic characteristics of a *Bradyrhizobium* population recovered from a pasture soil. Applied and Environmental Microbiology 60, 1754–1761.
Bromfield, E.S.P., Tambong, J.T., Cloutier, S., Prevost, D., Laguerre, G., van Berkum, P., Thi, T.V.T., Assabgui, R., Barran, L.R., 2010. *Ensifer, Phyllobacterium* and *Rhizobium* species occupy nodules of *Medicago sativa* (alfalfa) and *Melilotus alba* (sweet clover) grown at a Canadian site without a history of cultivation. Microbiology 156, 505–520.
Chen, W.M., de Faria, S.M., Straliotto, R., Pitard, R.M., Simoes-Araujo, J.L., Chou, J.F., Chou, Y.J., Barrios, E., Prescott, A.R., Elliott, G.N., Sprent, J.I., Young, J.P.W., James, E.K., 2005. Proof that *Burkholderia* strains form effective symbioses with legumes: a study of novel mimosa-nodulating strains from South America. Applied and Environmental Microbiology 71, 7461–7471.
Chen, W.M., Moulin, L., Bontemps, C., Vandamme, P., Bena, G., Boivin-Masson, C., 2003. Legume symbiotic nitrogen fixation by beta-proteobacteria is widespread in nature. Journal of Bacteriology 185, 7266–7272.
Cooper, J.E., 2007. Early interactions between legumes and rhizobia: disclosing complexity in a molecular dialogue. Journal of Applied Microbiology 103, 1355–1365.
Dedysh, S.N., Ricke, P., Liesack, W., 2004. NifH and NifD phylogenies: an evolutionary basis for understanding nitrogen fixation capabilities of methanotrophic bacteria. Microbiology-SGM 150, 1301–1313.
Deng, Z.S., Zhao, L.F., Kong, Z.Y., Yang, W.Q., Lindström, K., Wang, E.T., Wei, G.H., 2011. Diversity of endophytic bacteria within nodules of the *Sphaerophysa salsula* in different regions of Loess Plateau in China. FEMS Microbiology Ecology 76, 463–475.
Depret, G., Laguerre, G., 2008. Plant phenology and genetic variability in root and nodule development strongly influence genetic structuring of *Rhizobium leguminosarum* biovar viciae populations nodulating pea. New Phytologist 179, 224–235.
Diouf, D., Fall, D., Chantreuil, C., Ba, A.T., Dreyfus, B., Neyra, M., Ndoye, I., Moulin, L., 2010. Phylogenetic analyses of symbiotic genes and characterization of functional traits of *Mesorhizobium* spp. strains associated with the promiscuous species *Acacia seyal* Del. Journal of Applied Microbiology 108, 818–830.
Dobert, R.C., Brei, B.T., Triplett, E.W., 1994. DNA-sequence of the common nodulation genes of *Bradyrhizobium elkanii* and their phylogenetic relationship to those of other nodulating bacteria. Molecular Plant–Microbe Interactions 7, 564–572.
Downie, J.A., 2010. The roles of extracellular proteins, polysaccharides and signals in the interactions of rhizobia with legume roots. FEMS Microbiology Reviews 34, 150–170.
Dresler-Nurmi, A., Fewer, D.P., Räsänen, L.A., Lindström, K., 2007. The diversity and evolution of rhizobia. Microbiology Monographs.
Eardly, B.D., Young, J.P.W., Selander, R.K., 1992. Phylogenetic position of *Rhizobium* sp strain or-191, a symbiont of both *Medicago sativa* and *Phaseolus vulgaris*, based on partial sequences of the 16s ribosomal-RNA and *nifH* genes. Applied and Environmental Microbiology 58, 1809–1815.
Estrella, M.J., Munoz, S., Soto, M.J., Ruiz, O., Sanjuan, J., 2009. Genetic diversity and host range of rhizobia nodulating *Lotus tenuis* in typical soils of the Salado river basin (Argentina). Applied and Environmental Microbiology 75, 1088–1098.
Farida, B., Géraldine, D., Abdelghani, B., Djellali, B., Said, B., Gisèle, L., 2009. *Retama* species growing in different ecological-climatic areas of northeastern Algeria have a narrow range of rhizobia that form a novel phylogenetic clade within the *Bradyrhizobium* genus. Systematic and Applied Microbiology 32, 245–255.
Flores, M., Gonzalez, V., Pardo, M.A., Leija, A., Martinez, E., Romero, D., Pinero, D., Davila, G., Palacios, R., 1988. Genomic instability in *Rhizobium phaseoli*. Journal of Bacteriology 170, 1191–1196.
Fred, E.B., Baldwin, I.L., McCoy, E., 1932. Root Nodule Bacteria and Leguminous Plants. University of Wisconsin Press, Madison.
Freitas, A.D.S., Sampaio, E., Santos, C., Fernandes, A.R., 2010. Biological nitrogen fixation in tree legumes of the Brazilian semi-arid caatinga. Journal of Arid Environments 74, 344–349.
Gaunt, M.W., Turner, S.L., Rigottier-Gois, L., Lloyd-Macgilp, S.A., Young, J.P.W., 2001. Phylogenies of *atpD* and *recA* support the small subunit rRNA-based classification of rhizobia. International Journal of Systematic and Evolutionary Microbiology 51, 2037–2048.
Gevers, D., Huys, G., Swings, J., 2001. Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. FEMS Microbiology Letters 205, 31–36.
Han, T.X., Han, L.L., Wu, L.J., Chen, W.F., Sui, X.H., Gu, J.G., Wang, E.T., Chen, W.X., 2008. *Mesorhizobium gobiense* sp. nov. and *Mesorhizobium tarimense* sp. nov., isolated from wild legumes growing in desert soils of Xinjiang, China. International Journal of Systematic and Evolutionary Microbiology 58, 2610–2618.
Haukka, K., Lindström, K., Young, J.P.W., 1998. Three phylogenetic groups of *nodA* and *nifH* genes in *Sinorhizobium* and *Mesorhizobium* isolates from leguminous trees growing in Africa and Latin America. Applied and Environmental Microbiology 64, 419–426.
Hou, B.C., Wang, E.T., Li, Y., Jia, R.Z., Chen, W.F., Man, C.X., Sui, X.H., Chen, W.X., 2009. Rhizobial resource associated with epidemic legumes in Tibet. Microbial Ecology 57, 69–81.
Howieson, J.G., O'Hara, G.W., Carr, S.J., 2000. Changing roles for legumes in Mediterranean agriculture: developments from an Australian perspective. Field Crops Research 65, 107–122.
Hynes, M.F., Oconnell, M.P., 1990. Host plant effect on competition among strains of *Rhizobium leguminosarum*. Canadian Journal of Microbiology 36, 864–869.
Ibáñez, F., Angelini, J., Taurian, T., Tonelli, M.L., Fabra, A., 2009. Endophytic occupation of peanut root nodules by opportunistic Gammaproteobacteria. Systematic and Applied Microbiology 32, 49–55.
Jarabo-Lorenzo, A., Pérez-Galdona, R., Donate-Correa, J., Rivas, R., Velázquez, E., Hernández, M., Temprano, F., Martínez-Molina, E., Ruiz-Argüeso, T., León-Barrios, M., 2003. Genetic diversity of Bradyrhizobial populations from diverse geographic origins that nodulate *Lupinus* spp. and *Ornithopus* spp. Systematic and Applied Microbiology 26, 611–623.
Jarvis, B.D.W., VanBerkum, P., Chen, W.X., Nour, S.M., Fernandez, M.P., CleyetMarel, J.C., Gillis, M., 1997. Transfer of *Rhizobium loti, Rhizobium huakuii, Rhizobium ciceri, Rhizobium mediterraneum*, and *Rhizobium tianshanense* to *Mesorhizobium* gen. nov. International Journal of Systematic Bacteriology 47, 895–898.
Kan, F.L., Chen, Z.Y., Wang, E.T., Tian, C.F., Sui, X.H., Chen, W.X., 2007. Characterization of symbiotic and endophytic bacteria isolated from root nodules of herbaceous legumes grown in Qinghai-Tibet plateau and in other zones of China. Archives of Microbiology 188, 103–115.
Lafay, B., Burdon, J.J., 2006. Molecular diversity of rhizobia nodulating the invasive legume *Cytisus scoparius* in Australia. Journal of Applied Microbiology 100, 1228–1238.
Lambinon, J., Langhe, J.D., Delvosalle, L., Duvigneaud, J., 1998. Flora van België, het Groothertogdom Luxemburg, Noord-Frankrijk en de Aangrenzende Gebieden, Derde ruk ed. De Nationale Plantentuin van België, Meise.
Lorite, M.J., Donate-Correa, J., del Arco-Aguilar, M., Galdona, R.P., Sanjuan, J., Leon-Barrios, M., 2010. *Lotus* endemic to the Canary Islands are nodulated by diverse and novel rhizobial species and symbiotypes. Systematic and Applied Microbiology 33, 282–290.
Maâtallah, J., Berraho, E.B., Muñoz, S., Sanjuan, J., Lluch, C., 2002. Phenotypic and molecular characterization of chickpea rhizobia isolated from different areas of Morocco. Journal of Applied Microbiology 93, 531–540.
Martens, M., Delaere, M., Coopman, R., De Vos, P., Gillis, M., Willems, A., 2007. Multilocus sequence analysis of *Ensifer* and related taxa. International Journal of Systematic and Evolutionary Microbiology 57, 489–503.
Masson-Boivin, C., Giraud, E., Perret, X., Batut, J., 2009. Establishing nitrogen-fixing symbiosis with legumes: how many rhizobium recipes? Trends in Microbiology 17, 458–466.
Menna, P., Barcellos, F.G., Hungria, M., 2009. Phylogeny and taxonomy of a diverse collection of *Bradyrhizobium* strains based on multilocus sequence analysis of the 16S rRNA gene, ITS region and *glnII, recA, atpD* and *dnaK* genes. International Journal of Systematic and Evolutionary Microbiology 59, 2934–2950.
Merabet, C., Martens, M., Mahdhi, M., Zakhia, F., Sy, A., Le Roux, C., Domergue, O., Coopman, R., Bekki, A., Mars, M., Willems, A., de Lajudie, P., 2010. Multilocus sequence analysis of root nodule isolates from *Lotus arabicus* (Senegal), *Lotus creticus, Argyrolobium uniflorum* and *Medicago sativa* (Tunisia) and description of *Ensifer numidicus* sp. nov. and *Ensifer garamanticus* sp. nov. International Journal of Systematic and Evolutionary Microbiology 60, 664–674.
Mergaert, P., Van Montagu, M., Holsters, M., 1997. Molecular mechanisms of Nod factor diversity. Molecular Microbiology 25, 811–817.
Mierzwa, B., Wdowiak-Wrobel, S., Kalita, M., Gnat, S., Malek, W., 2010a. Insight into the evolutionary history of symbiotic genes of *Robinia pseudoacacia* rhizobia deriving from Poland and Japan. Archives of Microbiology 192, 341–350.
Mierzwa, B., Wdowiak-Wrobel, S., Maek, W., 2009. Phenotypic, genomic and phylogenetic characteristics of rhizobia isolated from root nodules of *Robinia pseudoacacia* (black locust) growing in Poland and Japan. Archives of Microbiology 191, 697–710.
Mierzwa, B., Wdowiak-Wrobel, S., Malek, W., 2010b. *Robinia pseudoacacia* in Poland and Japan is nodulated by *Mesorhizobium amorphae* strains. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 97, 351–361.
Mnasri, B., Badri, Y., Saidi, S., de Lajudie, P., Mhamdi, R., 2009. Symbiotic diversity of *Ensifer meliloti* strains recovered from various legume species in Tunisia. Systematic and Applied Microbiology 32, 583–592.
Mnasri, B., Mrabet, M., Laguerre, G., Aouani, M.E., Mhamdi, R., 2007. Salt-tolerant rhizobia isolated from a Tunisian oasis that are highly effective for symbiotic N_2-fixation with *Phaseolus vulgaris* constitute a novel biovar (bv. mediterranense) of *Sinorhizobium meliloti*. Archives of Microbiology 187, 79–85.
Moulin, L., Bena, G., Boivin-Masson, C., Stepkowski, T., 2004. Phylogenetic analyses of symbiotic nodulation genes support vertical and lateral gene co-transfer within the *Bradyrhizobium* genus. Molecular Phylogenetics and Evolution 30, 720–732.
Muresu, R., Maddau, G., Delogu, G., Cappuccinelli, P., Squartini, A., 2010. Bacteria colonizing root nodules of wild legumes exhibit virulence-associated properties of mammalian pathogens. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 97, 143–153.
Mutch, L.A., Young, J.P.W., 2004. Diversity and specificity of *Rhizobium leguminosarum* biovar viciae on wild and cultivated legumes. Molecular Ecology 13, 2435–2444.
Palaniappan, P., Chauhan, P.S., Saravanan, V.S., Anandham, R., Sa, T.M., 2010. Isolation and characterization of plant growth promoting endophytic bacterial isolates from root nodule of *Lespedeza* sp. Biology and Fertility of Soils 46, 807–816.
Pankhurst, C.E., Craig, A.S., Jones, W.T., 1979. Effectiveness of *Lotus* root-nodules. 1. Morphology and flavolan content of nodules formed on *Lotus pedunculatus* by fast-growing *Lotus* rhizobia. Journal of Experimental Botany 30, 1085–1093.
Pearson, W.R., 1990. Rapid and sensitive sequence comparison with Fastp and Fasta. Methods in Enzymology 183, 63–98.

Perez-Fernandez, M.A., Lamont, B.B., 2003. Nodulation and performance of exotic and native legumes in Australian soils. Australian Journal of Botany 51, 543–553.
Perret, X., Staehelin, C., Broughton, W.J., 2000. Molecular basis of symbiotic promiscuity. Microbiology and Molecular Biology Reviews 64, 180–201.
Radeva, G., Jurgens, G., Niemi, M., Nick, G., Suominen, L., Lindström, K., 2001. Description of two biovars in the *Rhizobium galegae* species: biovar orientalis and biovar officinalis. Systematic and Applied Microbiology 24, 192–205.
Ramirez-Bahena, M.H., Velazquez, E., Fernandez-Santos, F., Peix, A., Martinez-Molina, E., Mateos, P.F., 2009. Phenotypic, genotypic, and symbiotic diversities in strains nodulating clover in different soils in Spain. Canadian Journal of Microbiology 55, 1207–1216.
Raymond, J., Siefert, J.L., Staples, C.R., Blankenship, R.E., 2004. The natural history of nitrogen fixation. Molecular Biology and Evolution 21, 541–554.
Rincon, A., Arenal, F., Gonzalez, I., Manrique, E., Lucas, M.M., Pueyo, J.J., 2008. Diversity of rhizobial bacteria isolated from nodules of the gypsophyte *Ononis tridentata* L. growing in spanish soils. Microbial Ecology 56, 223–233.
Rivas, R., Martens, M., de Lajudie, P., Willems, A., 2009. Multilocus sequence analysis of the genus *Bradyrhizobium*. Systematic and Applied Microbiology 32, 101–110.
Rogel, M.A., Ormeño-Orrillo, E., Martinez Romero, E., 2011. Symbiovars in rhizobia reflect bacterial adaptation to legumes. Systematic and Applied Microbiology 34, 96–104.
Sajnaga, E., Malek, W., Lotocka, B., Stepkowski, T., Legocki, A., 2001. The root-nodule symbiosis between *Sarothamnus scoparius* L. and its microsymbionts. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 79, 385–391.
Sarita, S., Sharma, P.K., Priefer, U.B., Prell, J., 2005. Direct amplification of rhizobial *nodC* sequences from soil total DNA and comparison to *nodC* diversity of root nodule isolates. FEMS Microbiology Ecology 54, 1–11.
Steenkamp, E.T., Stepkowski, T., Przymusiak, A., Botha, W.J., Law, I.J., 2008. Cowpea and peanut in southern Africa are nodulated by diverse *Bradyrhizobium* strains harboring nodulation genes that belong to the large pantropical clade common in Africa. Molecular Phylogenetics and Evolution 48, 1131–1144.
Suominen, L., Roos, C., Lortet, G., Paulin, L., Lindström, K., 2001. Identification and structure of the *Rhizobium galegae* common nodulation genes: evidence for horizontal gene transfer. Molecular Biology and Evolution 18, 907–916.
Sy, A., Giraud, E., Jourand, P., Garcia, N., Willems, A., de Lajudie, P., Prin, Y., Neyra, M., Gillis, M., Boivin-Masson, C., Dreyfus, B., 2001. Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. Journal of Bacteriology 183, 214–220.
Tamura, K., Peterson, D., Peterson, N., Stecher G., Nei, M., Kumar, S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parismony methods. Molecular Biology and Evolution, Advance Access published May 4, 2011. doi:10.1093/molbev/msr121.
Trujillo, M.E., Alonso-Vega, P., Rodriguez, R., Carro, L., Cerda, E., Alonso, P., Martinez-Molina, E., 2010. The genus *Micromonospora* is widespread in legume root nodules: the example of *Lupinus angustifolius*. The ISME Journal 4, 1265–1281.
Turner, S.L., Zhang, X.-X., Li, F.-D., Young, J.P.W., 2002. What does a bacterial genome sequence represent? Mis-assignment of MAFF 303099 to the genospecies *Mesorhizobium loti*. Microbiology 148, 3330–3331.
Ulrich, A., Zaspel, I., 2000. Phylogenetic diversity of rhizobial strains nodulating *Robinia pseudoacacia* L. Microbiology-Uk 146, 2997–3005.
Van Landuyt, W., Hoste, I., Vanhecke, L., Vercruysse, W., Van Den Bremt, P., De Beer, D., 2006a. Atlas van de Flora van Vlaanderen en het Brussels Gewest. INBO and Nationale Plantentuin van België, Brussels.
Van Landuyt, W., Vanhecke, L., Hoste, I., 2006b. Rode Lijst van de vaatplanten van Vlaanderen en het Brussels Hoofdstedelijk Gewest. In: Van Landuyt, W., Hoste, I., Vanhecke, L., Vercruysse, W., Van Den Bremt, P., De Beer, D. (Eds.), Atlas van de Flora van Vlaanderen en het Brussels Gewest. INBO and Nationale Plantentuin van België, Brussels.
Van Landuyt, W., Vanhecke, L., Hoste, I., Bauwens, D., 2011. Do the distribution patterns of vascular plant species correspond to biogeographical classifications based on environmental data? A case study from northern Belgium. Landscape and Urban Planning 99, 93–103.
Vancanneyt, M., Mengaud, J., Cleenwerck, I., Vanhonacker, K., Hoste, B., Dawyndt, P., Degivry, M.C., Ringuet, D., Janssens, D., Swings, J., 2004. Reclassification of *Lactobacillus kefirgranum* Takizawa et al. 1994 as *Lactobacillus kefiranofaciens* subsp. *kefirgranum* subsp. nov. and emended description of L-kefiranofaciens Fujisawa, et al. 1988. International Journal of Systematic and Evolutionary Microbiology 54, 551–556.
Vandamme, P., Goris, J., Chen, W.M., de Vos, P., Willems, A., 2002. *Burkholderia tuberum* sp. nov. and *Burkholderia phymatum* sp. nov., nodulate the roots of tropical legumes. Systematic and Applied Microbiology 25, 507–512.
Velazquez, E., Valverde, A., Rivas, R., Gomis, V., Peix, A., Gantois, I., Igual, J.M., Leon-Barrios, M., Willems, A., Mateos, P.F., Martinez-Molina, E., 2010. Strains nodulating *Lupinus albus* on different continents belong to several new chromosomal and symbiotic lineages within *Bradyrhizobium*. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 97, 363–376.
Versalovic, J., Schneider, M., De Bruijn, F.J., Lupski, J.R., 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods in Molecular and Cellular Biology 5, 25–40.
Villegas, M.D., Rome, S., Maure, L., Domergue, O., Gardan, L., Bailly, X., Cleyet-Marel, J.C., Brunel, B., 2006. Nitrogen-fixing sinorhizobia with *Medicago laciniata* constitute a novel biovar (bv. medicaginis) of *S. meliloti*. Systematic and Applied Microbiology 29, 526–538.
Vincent, J.M., 1970. A Manual for the Practical Study of the Root-nodule Bacteria IBP Handbook 15.
Vinuesa, P., Leon-Barrios, M., Silva, C., Willems, A., Jarabo-Lorenzo, A., Perez-Galdona, R., Werner, D., Martinez-Romero, E., 2005. *Bradyrhizobium canariense* sp. nov., an acid-tolerant endosymbiont that nodulates endemic genistoid legumes (Papilionoideae: Genisteae) from the Canary Islands, along with *Bradyrhizobium japonicum* bv. genistearum, *Bradyrhizobium* genospecies alpha and *Bradyrhizobium* genospecies beta. International Journal of Systematic and Evolutionary Microbiology 55, 569–575.
Wdowiak-Wrobel, S., Malek, W., 2010. Following phylogenetic tracks of *Astragalus cicer* microsymbionts. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 97, 21–34.
Wei, G.H., Chen, W.M., Zhu, W.F., Chen, C., Young, J.P.W., Bontemps, C., 2009. Invasive *Robinia pseudoacacia* in China is nodulated by *Mesorhizobium* and *Sinorhizobium* species that share similar nodulation genes with native American symbionts. FEMS Microbiology Ecology 68, 320–328.
Weir, B.S., Turner, S.J., Silvester, W.B., Park, D.C., Young, J.A., 2004. Unexpectedly diverse *Mesorhizobium* strains and *Rhizobium leguminosarum* nodulate native legume genera of New Zealand, while introduced legume weeds are nodulated by *Bradyrhizobium* species. Applied and Environmental Microbiology 70, 5980–5987.
Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16s ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology 173, 697–703.
Wielbo, J., Marek-Kozaczuk, M., Mazur, A., Kubik-Komar, A., Skorupska, A., 2010. Genetic and metabolic divergence within a *Rhizobium leguminosarum* bv. trifolii population recovered from clover nodules. Applied and Environmental Microbiology 76, 4593–4600.
Willems, A., 2006. The taxonomy of rhizobia: an overview. Plant and Soil 287, 3–14.
Willems, A., Collins, M.D., 1993. Phylogenetic analysis of rhizobia and agrobacteria based on 16s ribosomal-RNA gene sequences. International Journal of Systematic Bacteriology 43, 305–313.
Zakhia, F.R., Jeder, H., Domergue, O., Willems, A., Cleyet-Marel, J.C., Gillis, M., Dreyfus, B., de Lajudie, P., 2004. Characterisation of wild legume nodulating bacteria (LNB) in the infra-arid zone of Tunisia. Systematic and Applied Microbiology 27, 380–395.